



## MOLECULAR CHARACTERIZATION OF GENE 16S rRNA MICRO SYMBIONTS IN SPONGE AT MELAWAI BEACH, EAST KALIMANTAN

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### ABSTRACT

Molecular characterization studies have been conducted 16S rRNA gene micro symbiont of sponge origin Melawai Beach, Balikpapan in East Kalimantan. Objective analysis of histo-morphological research, isolation-purification, molecular characterization of micro-symbiont genes in order to search symbiont bacteria that can live in extreme environments contaminated hydrocarbon waste. The research method that morphological identification, isolation-purification and molecular characterization of the 16S rRNA gene with Chain Reaction Polymerization method. The results of histo-morphological analysis concluded sponge samples with species of *Callyspongia* sp. Isolation and purification mikro symbionts of sponge obtained 2 (two) isolates. Characteristics of Isolates 1; spherical shape, colonize and creamy, while isolates 2; jagged shape, oval and white colonies. Molecular characterization of the 16S rRNA gene by PCR, *Bacillus subtilis* strain BAB-684 identification for isolates one is the number of nucleotide pairs reached 899 bp and the degree of similarity in GenBank reached 89% homologous, while the second is a *Bacillus flexus* strain PHCDB20 isolates the number reached 950 bp nucleotide pairs with the degree of similarity in GenBank reached 99% homologous.

Keywords: *Callyspongia* sp, micro-symbionts, characterization, gene Molecules

### INTRODUCTION

The marine environment has a vital role in human life, not only as a means of transportations, over the sea is a wide variety of organisms living and needed to ensure the survival of organisms, including humans. Many findings mineral deposits of minerals, oil and gas in the sea where the sea adds vital to human life.

Potential contamination of waste petroleum hydrocarbons can occur in almost all lines of petroleum activities ranging from exploration through refining process and potentially generate sludge waste of petroleum (*oil sludge*), (Aliyanta *et al.*, 2011).

The main components of petroleum contaminants sludge are aliphatic and aromatic hydrocarbons (PAH) are hydrophobic, making it easy to stick to

organic material of solid particles formed mikropolutan persistent in the environment. PAHs as toxic substances can damage marine life and human health due to potentially interfere with carcinogenic and mutagenic effects have, (Gan *et al.*, 2009; Wu *et al.* 2008).

Human responsibility in defending the quality of the marine environment is done by controlling pollution petroleum waste either physics, chemistry or biology has been conducted on an ongoing basis. One of the efforts to address waste oil contaminated environment is the use of microorganisms that can degrade components of toxic hydrocarbons PAHs, (Syakti *et al.*, 2013; Gan *et al.*, 2009).

One of the microorganisms that can degrade hydrocarbons PAHs particularly

low and high molecular weight are bacteria, (Teng *et al.*, 2010; Gan *et al.*, 2009; Avecedo *et al.*, 2011).

Some marine bacteria known to degrade aromatic hydrocarbon compounds with high toxicity by utilizing components of PAH as a source of carbon and energy source, (Gan *et al.*, 2009; Murniasih *et al.*, 2009), is the dominant type of bacteria *Enterobacter* sp., *Bacillus* sp., *Clostridium* sp., *Pseudomonas* sp., *Aeromonas* sp and *Citrobacter* sp., (Gan *et al.*, 2009; Crawford in of Yani and Akbar, 2012; Ekpo and Udofia, 2008).

Degrading bacterium *Bacillus* group component aromatic compounds, such as: *B. macerans*, *B. mycoides*, *B. megaterium*, *B. subtilis*, *B. pumilus*, *B. thermoleovorans*, *B. gordonae*, and *B. benzoovorans*, (Gan *et al.*, 2009; Zarilla, 1987).

Some of the above mentioned bacteria can be symbiotic with sponges, (Murniasih, 2010; Mahdiyah *et al.*, 2012). Bacteria is one of the microorganisms that are often found in symbiosis with the sponge. It is estimated that up to 40% biomass several types of sponges prepared by the bacterial community, (Thakur, 2004). Identified several indigenous bacteria can degrade petroleum sludge, (Syakti *et al.*, 2013).

Symbiont microorganisms with sponge has two important roles in biological systems sponge, first as a source of good food by microorganisms symbionts itself or by sponge, both micro sponge symbionts make as host for life and utilize micro sponge simbiotnya as a defense in order to adapt and live in the environment extreme (polluted environments), (Steindler *et al.*, 2002; Zarilla, 1987).

Sponges are multicellular metazoa animals, which are filter feeder porous, so it becomes a habitat for microorganisms to nesting in the body, (Kozloff, 1990).

Melawai beach Balikpapan including the tides beach, is a tourist destination and culinary visited by many people. Melawai Beach scenery is quite interesting because it is a means to view traffic tankers, cargo and vessels passing the Pelni, this situation

happens every day, which contributed an increase in the volume of oil spills, (Jayardana, 2006). The circumstances which encourage the search and identify research sponge that can live in polluted areas are also very possible hydrocarbon presence of micro-symbionts that can detoxify waste toksit properties of hydrocarbons.

The purpose of this research is the search and micro sponge symbionts that can degrade PAHs in particular hydrocarbon waste through histomorphological analysis, isolation-purification and molecular characterization of the 16S rRNA gene micro symbiont of sponge origin Melawai Beach Balikpapan East Kalimantan. The benefits to be achieved from this study are expected to contribute and can provide scientific information about the type of sponge and symbiotic bacteria can live in areas contaminated by waste oil to help reduce the negative impact that the toxic properties of marine life damage can be minimized.

## METHODS

### Material

Sponges origin Melawai Beach Balikpapan, CH<sub>3</sub>OH pa, sterile sea water, Phosphate Buffer Saline (PBS), Sea Water Complit (SWC), Media NA, marine agar (MA), 25 % glycerol, micro-symbionts sponge isolates, 4 % formalin, Aquabides, ddH<sub>2</sub>O, chelex 20%, a pair of universal primary sequences of the 16S rRNA gene of *E. coli*: FP-U1 (5'-CCAGCAGCCGCGGTAATACG-3') at nucleo-tides 518-537, and RP-U2 is (5'-ATCGG (C/T) TACCTTGTTACGACTTC-3') corresponding to nucleotides 1513-1491, template DNA, Taq DNA polymerase (Perkin-Elmer, Norwalk, Conn), PCR Mix, Triton X-100, Tris, EDTA, HCl, KCl, MgCl<sub>2</sub>, paraffin, deoxynucleoside triphosphate, polyacrylamide gel, agarose, Ethidium Bromide.

### Instrument

Scuba, underwater camera, GPS,

dissectingpan, scalpel, forceps, wattle, plastic bag, ice box, haemositometer, porcelain cup, mortar and pestle, blender, set of glassware, bunsen, analytical balance, hot plate, rubber suction, Whatman, oven, freezer, BOD bottles, thermometers, ose round, effendorf 1.5 mL tube, vortex shaker, centrifuged 1000 rpm, 4000 rpm and 10,000 rpm, media spoon, stirrer, compactor container gel, universal pH paper, salinometer, stop watch, laminary water flow (LAF), autoclave, filter 0.2 lm, PCR machine (Biorad), Bioedit program, MAS-100 (Microbio-logical Air Sampler), micropipette, tip filter, gene cycler, gel doc, and electrophoresis.

### Time and Place Research

When the study was conducted in July to December 2014. The research at the Laboratory of Pharmaceutical Microbiology Faculty of Pharmacy, University of Hasanuddin, and Microbiology Laboratory Hasanuddin University Educational Hospital.

### Samples and Sampling Location

Potential sponge samples obtained from petroleum hydrocarbon contaminated area, Melawai Beach Balikpapan in East Kalimantan., (Jayardana, 2006). Sponge samples were taken at four points with code samples A, B, C, D, taken at a depth of 2.7 m with scuba diving technique was held on December 19, 2013



Figure 1. Sampling point sponge of Melawai Beach

After the sponge samples lifted to the surface, immediate photo shoot and taken most of sponge tissue, then put in a 5 % marine broth medium for isolated (direct plating method). Supporting data measured at each sampling point is: the coordinates, temperature, depth, salinity, and pH.

### Analysis Method

The method of analysis is done in three (3) stages of the work, namely: 1) histo-morphological analysis of sponge; 2) isolation and purification of micro-symbionts; 3) molecular characterization of the 16S rRNA gene PCR method.

### Analysis morphology of the sponge

Identification of the sponge is done by analyzing the specific characteristics (color, texture, shape, size and depth of sampling. While the histo-morphological analysis of sponge cells under a microscope by MTT assay, assay, (A) Cell Culture Before treatment 24 h incubation, after 4 hours addition of MTT reagent, (B) control cells, (C) the extract concentration of 960 mg / mL, (D) extract concentration of 7.5 mg / mL, (E) control DMSO, (Hooper, 1997).

### Isolation and purification of bacterial symbionts sponge

Sea sponge that has been identified in the histo-morphological analysis, then taken to be isolated by means of a sponge sample was sprayed on the surface with sterile sea water with every 1 ratio of: 5 (cm<sup>2</sup>: mL) sterile sea water, so that only bacteria with a strong affinity separate. Part mesohyl taken with  $\pm 1 \times 1$  cm size, crushed and diluted with Phosphate Buffer Saline (PBS) sterile with a ratio of 1: 1, (Kim *et al.*, 2006).

Isolation of bacteria using a sterile swab, (Wahl *et al.*, 1994), which is rubbed with a direction on the outer surface of the sponge. Sterile swab was rubbed on the surface of the sample is introduced into the dilution tube containing sterile PBS and divorteks. Dispersed dilution results in a petri dish that already contains media Sea

Water Complit (SWC) with 1 liter of medium composition consisting of 5 g/L bacto peptone, 1 g/L yeast extract and 3 mL/L glycerol, and incubated at 30 °C for 24-36 hours, the observed growth of the colony. Each colony morphology observed growing bacteria, such as color, size and shape of the colony. Colonies that grew dominant for isolated and purified separated using the same media.

In the direct plating method, observed colonies that grow after incubation for 1 to 2 weeks. Colonies that shapes and different colors isolated. Each colony is done purification by means of scraping 1 ose colonies a zigzag direction on a petri dish containing media 100 % marine so then incubated at 30 °C for 1-2 days. After the new colony grew and then re etching performed on 100 % marine media in order to obtain a single colony.

Enrichment method by distributing the liquid culture medium to 100 % marine order, made after 2 weeks of incubation, then the culture was incubated at 30 °C for one week and the number of colonies counted, isolated and purified. Storage of purified performed using 25 % glycerol in the medium MA

### Characterization of the 16S rRNA gene molecules

PCR is an in vitro method to produce a number of specific DNA fragments with a length and a predetermined amount the squens of a small number of complex template, (Davis, 1994). Deocsiribosa Nucleic Acid (DNA) of pure isolates were extracted with Chelex method 20 %, and then carried through 35 cycles of PCR, denatured at 95 ° C, 1 min, annealing at a temperature of 55 °C: 1 min and extension at 72 °C: 2 minutes, incubated at 72 °C: 10 minutes, (Alamri, 2010). DNA electrophoresis, PCR products with clear tape size of 150 bp. (Clarridge, 2004; Ramadhan *et al.*, 2012)

DNA sequencing is done by sending to Macrogen, the sequencing results to the GenBank identified through BLAST (Basic

Local Alignment Search Tool), or a multiple alignment with GenBank under Bioedit program version 7.2.5. (Murayama *et al.*, 2010)

## RESULTS AND DISCUSSION

### Data sampling

Supporting data observed when sampling sponge in Melawai Beach of Balikpapan is presented in Table 1, below:

Table1. Conditions Melawai Beach of Balikpapan as Water Sampling Points

The Parameter	Value Measurement
Coordinates	01 <sup>0</sup> 16. 36'8" LS
sampling	116 <sup>0</sup> 48. 23'6" BT
Temperature (°C)	29
Salinity (ppt)	30 ‰
pH	7
Depth of sampling	2,7 m

Primary Data: Januari, 2014

Physical characteristics sponge samples directly observed in the field, beige brown, elongated shape partially clustered, have spicules form fibrous silicate and sponging. massiva sponge growth form and oscula scattered along the surface of the sponge body, skeleton irregular and contains spongin consists of the main part and stringy parts. Based on this it was concluded that the sponges are identified by species *Callyspongia* sp, family *Callyspongiidae*, class *Demospongiae*, (Duchassaing & Michelotti, 1964 in Dactylia, 1885; Marzuki *et al.*, 2014; Ismet *et al.*, 2011; Thakur, 2004).

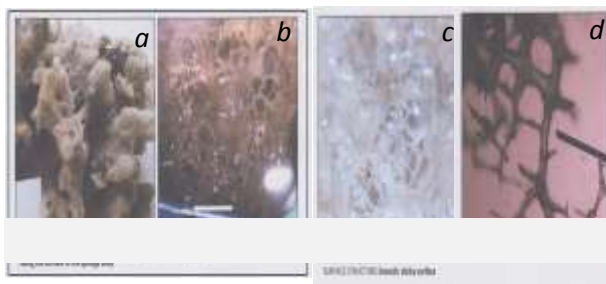


Figure 2. Results of analysis of the morphology of the sponge species *Callyspongia* sp., A) growth form massiva, oscula scattered along the surface of the sponge body; b) the results of staining with EtOH; c) the results compress texture; d) megascleres Axia be tangent with various sizes. (Primary Data, September 2014).

These results are in accordance conclusion, Ruppert *et al.*, 2004; Gazave, 2010; Ismet *et al.*, 2011, which states that the structure of the sponge at least have skeleton contain debris or spicules and spongin, sponge cells and possibly some micro-symbionts.

### Results Isolation and purification of bacterial symbionts sponge of *Callyspongia* sp.

Bacterial colonies were isolated and purification is growing colonies dominant. Both colonies identified can grow well in dilution  $10^{-2}$  s/ d  $10^{-4}$ .

Macroscopic identification and purification of the isolated sponge *Callyspongia* sp. obtained only two isolates. The first isolates with characteristics of large round shape and creamy-white, hereinafter referred isolates 1, and isolates both the characteristics of the jagged shape, elongated, brownish white, hereinafter referred to isolate 2, (Hooper, 1997; Mapstone, 1990).

The results of the isolation and purification of bacterial symbionts obtained only two isolates, it is reasonable and consistent with the observation of cell biomass was dominated by the skeleton

(spicules and cell debris), then the cell sponge and a little pellet of bacteria as well as morphological observation *Callyspongia* sp of sponge, (Silberhorn *et al.*, 2007; Ismet *et al.*, 2011; Murniasih *et al.*, 2010; Marzuki *et al.*, 2014)

### Molecular characterization of genes 16S rRNA isolate Micro symbionts

There are four stages of DNA isolation, first. cell solution; the second. DNA extraction; the third. DNA precipitation; and fourth. DNA leaching.

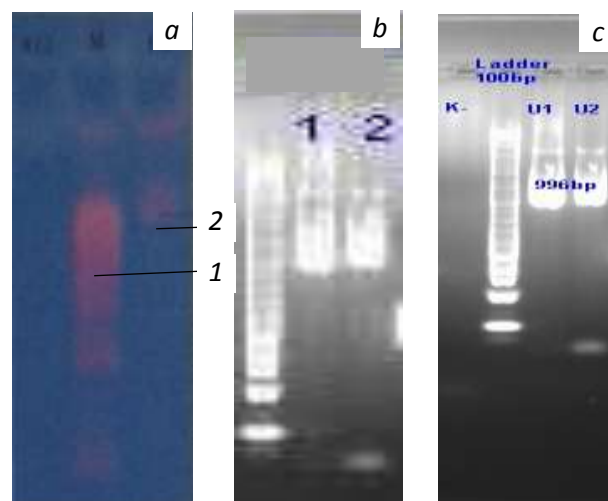


Figure 3. a) Results Genomic DNA isolation, number 1 genome comparison. 2 Genome number of samples; b) the results of genome amplification, the number is 1 estimates base pair (bp) isolates 1 and number 2 isolates 2; c) Results of amplification after purification with an estimated 996 bp bacterial symbionts sponge of *Callyspongia* sp, Primary Data, November 2014.

PCR with 16S rRNA gene sequencing allows the identification of bacteria is more accurate than those obtained by testing the phenotype. Analysis of 16S rRNA allows the discovery of new pathogens, as it can identify the bacteria that can not be cultured, (Samaila *et al.*, 2014).



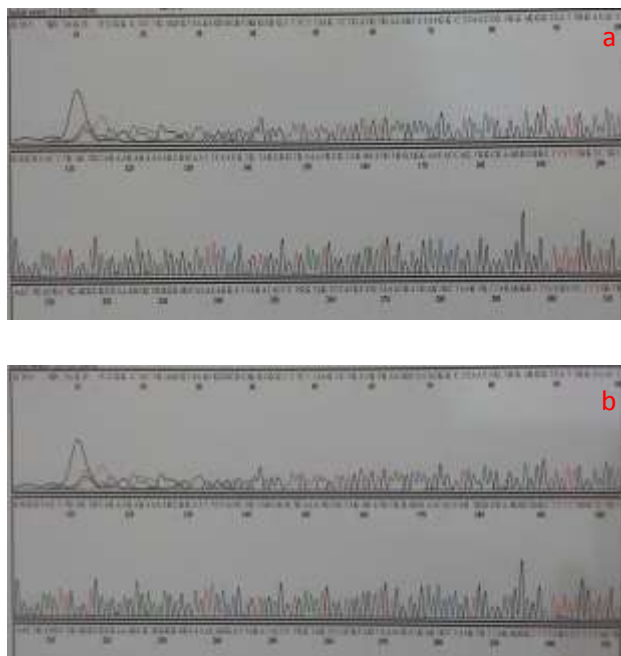


Figure 4. Chromatogram pieces of DNA sequences of bacterial symbionts *Callyspongia* sp isolates, a) isolate one piece of 17-230 bp sequences, b) Isolate 2 pieces of 10-205 bp sequence

Sequencing results obtained in the form of raw data, then processed using the software program Bioedit version 7.2.5. Data that has been processed by the program Bioedit used as baseline data to be processed back to the multiple alignment (multiple alignment) with the database. Universal 16S rRNA gene in bacteria, in general comparison of 16S rRNA sequences, (Clarridge, 2004).

Sequencing was opened through Bioedit program, then the bacterial DNA sequences inserted into the Basic Local Alignment program Search Tool, (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), sequences identified by GenBank DNA database on the site, (Samaila *et al.*, 2014; Clarridge, 2004).

Table 2 Results of BLAST (Basic Local Alignment Search Tool) the Micro symbionts of sponge

Sample code	Sample Sequences	Sequences GenBank	percent homologous (%)	Species
Isolate 1	17-916	554-1461	817/913 (89%)	<i>Bacillus subtilis</i>
Isolate 2	10-960	19-968	948/952 (99%)	<i>Bacillus flexus</i>

DNA sequence analysis of the sample sequences of isolates 1 on a stretch of nucleotides 17-916 or 899 bp, with details of the purine group consisting of 28.44 % Guanine, 24.27 % Adenine and pyrimidine group consisting of 25.10 % Cytosine, 21.15 % Thymine and there is a row of 1.04 % non pairs of nucleotides. These isolates have the same genus high reached 89 % homologous to the alignment of multiple sequences of GenBank at 554-1461 bp stretch. Then identified *Bacillus subtilis* strain BAB-684. Previously been conducted identifying *Basillus subtilis* PCR method at  $\pm 800$  bp, (Ling Xu *et al.*, 2012; Ramadhan *et al.*, 2012).

While the sequence of the DNA sequences of isolates 2 on the stretch of nucleotides 10-960 or 950 bp, with details of the purine group, consisting of 30.62 % Guanine, 25.46 % Adenine and pyrimidine group consisting of 22.58 % Cytosine, 20.31 % Thymine and there is a row of 1.03 % non pairs of nucleotides. These isolates have the same genus high reached 99 % homologous to multiple sequence alignments on a stretch of 19-968 bp GenBank identified *Bacillus flexus* strain PHCDB20. Previously been done identification of *Basillus flexus* PCR method to  $\pm 1,000$  bp, (Alamri, 2012). Both of these species is the genus *Bacillus*, family *Bacillaceae* and *Bacilli* class. Character of a bacterium is determined by the composition of the constituent DNA and be decisive in the performance of hydrocarbon degrading bacteria, (Ling Xu *et al.*, 2012).

The molecular structure of deoxyribose nucleotide acid constituent components can be seen in Figure 5.

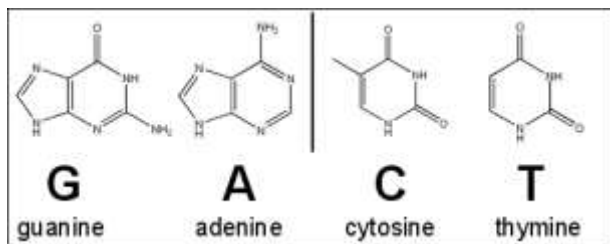


Figure 5. The structure of the DNA molecule

BLAST results that achieve a high degree of similarity genus when reaching over 80% homologous, (Ramadan *et al.*, 2012). Identification by PCR and DNA sequencing of bacteria through the BLAST program is more convincing as compared with bacterial DNA data in GenBank which contains tens of millions of existing bacterial DNA data, (Samaila *et al.* 2014).

These results provide a strong belief that these two species is the genus *Bacillus*, are known to carry out chemical reactions solving hydrocarbon molecules, (Teng *et al.*, 2010; Gan *et al.*, 2009; Aliyanta *et al.*, 2011; Syakti *et al.*, 2013), so it is natural that the *Callyspongia* sp symbioning sponges with *Basillus subtilis* and *Basillus flexus* viable and capable of adjusting to the hydrocarbon contaminated environments such as in Melawai Beach, Balikpapan, East Kalimantan

## CONCLUSION

Conclusions of research on the molecular characterization of the 16S rRNA gene micro symbiont of sponge origin waste hydrocarbon contaminated areas in Melawai Beach, Balikpapan, East Kalimantan, is the histo-morphological analysis obtained sponge with species of *Callyspongia* sp. The results of the isolation and purification of micro symbionts of sponge obtained 2 (two) isolates. Characteristics of Isolates 1, globular, colonize and creamy, while isolates 2, jagged shape, oval and white colonies. While the results of the molecular

characterization of the 16S rRNA gene by PCR using primers FP-U1 (5'-CG-CCAGCAGCCGCGGTAATA-3') at nucleoti-des 518-537, and RP-U2 (5'-ATCGG (C/T) TACCTTGTTACG-ACTTC- 3 '), to isolate one is bacteria species of *bacillus subtilis* strain BAB-684 the number of nucleotide pairs reached 899 bp and the degree of similarity in GenBank reached 89% homologous, while the isolates 2 are species of *bacillus flexus* strain PHCDB20 the number reached 950 bp nucleotide pairs with the degree of similarity in GenBank reached 99% homologous. Both species are micro symbiont of sponge *Callyspongia* sp obtained from hydrocarbon contaminated environments showed that the sponge and symbiotic bacteria capable of living and can degrade hydrocarbon waste.

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